Pestic. Sci. 1996, **48**, 359–373

Acylated 1,3-Aminopropanols as Repellents against Bloodsucking Arthropods†

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(Received 24 May 1996; revised version received 10 August 1996; accepted 21 August 1996)

Abstract: Starting from a set of known repellent compounds, a general structural framework with high probability for repellent activity was developed by molecular modelling techniques. Synthesis, structure–biological activity relationships of acylated 1,3-aminopropanols on the yellow fever mosquito *Aedes aegypti* and the properties and activity of the new development candidate compound KBR 3023 on *Ae. aegypti*, the malaria mosquito *Anopheles stephensi*, the common house mosquito *Culex quinquefasciatus* and the stable fly *Stomoxys calcitrans* are described. The sensory effect of KBR 3023 and deet has been studied in behavioural and neurophysiological investigations on *Ae. aegypti* and the American cockroach *Periplaneta americana*.

The compounds clearly reduce or even eliminate the approach behaviour towards attractants like host odours or sexual pheromones. Electrophysiological studies on the insects' olfactory receptor organs reveal that certain cell types, which are not involved in perception of the attractive odorants, respond to deet and/or KBR 3023. As soon as one of the compounds is presented together with an attractant, a new input is active in the brain, which adds to the input from other receptors activated by the attractant. This new overall pattern clearly differs from that elicited by the attractant, so that the insect is no longer able to detect the latter.

The specificity and mode of action of KBR 3023 was investigated by experiments exploring second-messenger responses elicited in antennal preparations of male *P. americana*. KBR 3023 induced a rapid increase in the concentration of inositol triphosphate in a dose-dependent and tissue-specific manner; other second-messenger systems were not affected. These observations suggest that KBR 3023 may act *via* subsets of G-protein-coupled receptors in sensory neurones.

Key words: repellents, synthesis, mosquitoes, cockroaches, molecular modelling, behaviour, electrophysiology, second messenger responses

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† One of a collection of papers on various aspects of pest control research contributed by staff of Bayer AG and collated by Dr M. Londershausen and Dr A. Turberg.

Pestic. Sci. 0031-613X/96/$09.00

1 INTRODUCTION

Bloodsucking arthropods transmit many diseases world-wide, the most important of these being malaria (*Plasmodium sp.*; vector *Anopheles sp.*)[1] in the tropics and Lyme disease (*Borrelia burgdorferi* s.l. (M. Johnson *et al.*); vector *Ixodes sp.* (L.)) in Europe and the USA.[2] Additionally, bites may cause skin irritation, allergic reactions and secondary infections.[3]

Among measures for personal protection against bloodsucking arthropods, repellents play an important role because they can be used at any place and time.[4] Beyond that, repellents are recommended by the WHO as means of protection against malaria because of the increasing resistance of *Plasmodium falciparum* (Welch) to anti-malarial drugs (e.g. chloroquin).[5] The most widely known and frequently employed compound is diethyl-*m*-toluamide (deet; **1**, Fig. 1) discovered in 1954,[6] which can be obtained in different formulations and which has a broad spectrum of efficacy against biting arthropods. It is superior to such other compounds as dimethyl phthalate (DMP; **2a**) against ticks of the species *Rhipicephalus sanguineus* Latreille and *Argas persicus* (Oken),[7] but not against *Ixodes scapularis* (Say) (= *Ixodes dammini*).[8] Against mosquitoes it shows better activity than **2a** against *Anopheles albimanus* (Wiedemann) and *An. farauti* (Laveran)[9] as well as against *Culex quinquefasciatus* (Say).[10] Tested against *Aedes aegypti* (L.), compound **1** performs better than **2a**, ethylhexanediol **3** or indalone **4**[11,12] as it does against *An. stephensi* (Liston), *An. gambiae* (Giles) and *An. freeborni* (Aitken), whereas **1** was inferior against *An. albimanus*.[13] Against *Phlebotomus papatasii* (Scopoli), **1** showed better efficacy than **2a**,[14] **3** or **4**.[15] The advantage of good efficacy is contrasted by some undesirable properties in that the compound causes irritation of skin and mucous membranes and is incompatible with some synthetic materials.[16] Furthermore, a certain neurotoxic potential has been described.[17–20]

Although many approaches have been undertaken to find new repellents only a few have led to new, more widely studied compounds, for example 3-(*N*-*n*-butyl-*N*-acetyl aminopropionic acid ethyl ester (Merck 3535) **5**,[16] *N*,*N*-diethyl-2-phenylacetamide (DEPA) **6**,[21] (2-Hydroxymethylcyclohexyl) acetic acid lactone (ClC-4) **7**[22] and *p*-menthane-3,8-diol **8**.[23]

It was the objective of this study to describe the synthesis and properties of new acylated 1,3-aminopropanols with effectiveness against the yellow fever mosquito *Ae. aegypti*, the malaria mosquito *An. ste-*

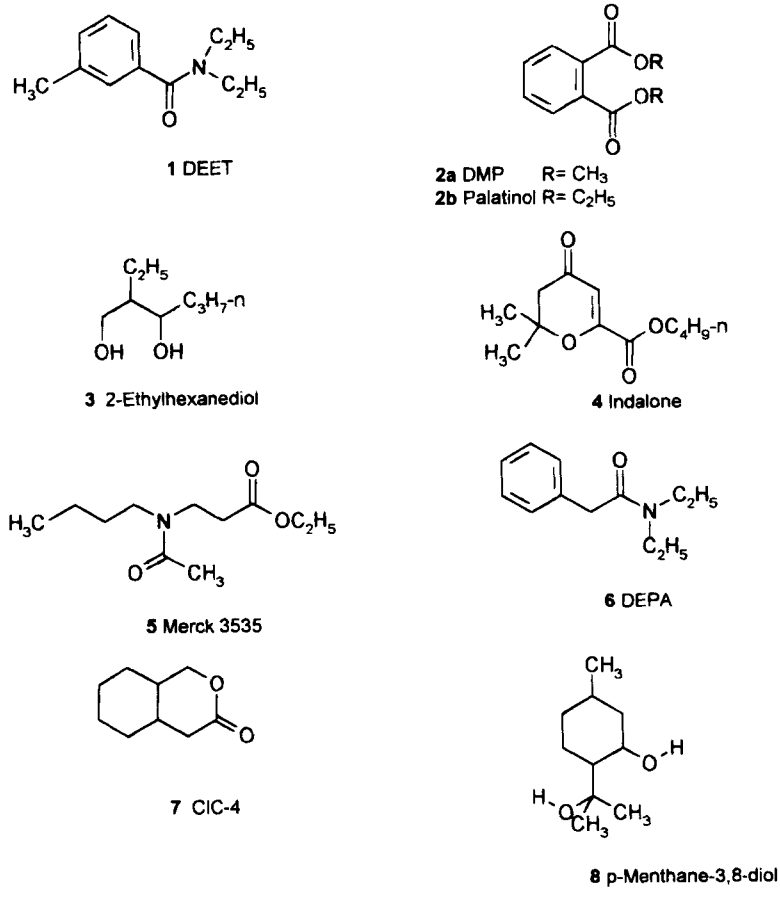

Fig. 1. Important commercial and experimental insect repellents.

phensi, the common house mosquito *C. quinquefasciatus* and the stable fly *Stomoxys calcitrans* L. comparable or superior to that of deet but without its disadvantages.

In order to find out more about the mechanisms by which these new compounds exert their aversive effects upon the target insects, we investigated behavioural and neurophysiological responses of two 'model' species from different groups of insects. Two contexts were chosen in which the compounds in question interfere with mainly odour-controlled behaviour: host-finding in female *Ae. aegypti*, and the detection of females by male American cockroaches *Periplaneta americana* (L.). This study includes quantitative measurements of behavioural responses and electrophysiological recordings from olfactory receptor organs.

To aid an understanding of the mechanism underlying recognition and transduction of these chemosensory active molecules, second-messenger cascades were analysed in antennal preparations from *P. americana*.

2 EXPERIMENTAL METHODS

2.1 Synthesis

All starting materials were obtained from Aldrich, Sigma or Bayer AG. The structures of the synthesised compounds were characterised by their proton resonance spectra in deuterochloroform and by their mass spectra. Sometimes rotamers were observed in the NMR spectra at room temperature.

Structures of important commercial and experimental insect repellents are in Fig. 1 and those of the compounds synthesised for this study are in Fig. 2. Data for test compounds of Types **9–12** (Fig. 2) are in Table 1.

*2.1.1 Acylation procedure for the synthesis of the amides **9a**, **11**, **12b** and **14** and the carbamates **9b** and **10**[24,25]*

2.1.1.1 Method a: A solution of 2-(2-hydroxyethyl)pyrrolidine or 2-(2-hydroxyethyl)piperidine (0·05 mole) and triethylamine (0·06 mole) in dry tetrahydrofuran (300 ml) was cooled to −20°C and the acid chloride or chloroformate (0·055 mole) was added dropwise. The reaction mixture was stirred for 18 h at 20°C, poured into ice-water (800 ml) and processed using standard methods. The oily residue was distilled at 0·1 mbar in a Kugelrohr apparatus.

For the synthesis of 2,- 3- or 4-hydroxyalkyl-*N*-alkylamides (**11**), 0·12 mole triethylamine and 0·11 mole of the acid chloride were used.

2.1.1.2 Method b: 2-Methylpropyl chloroformate (29 mmol) and sodium hydroxide solution (1 N; 30 ml) were dripped in parallel into a solution of (2-hydroxyethyl)piperidine (25 mmol) in toluene (30 ml) at 20°C.

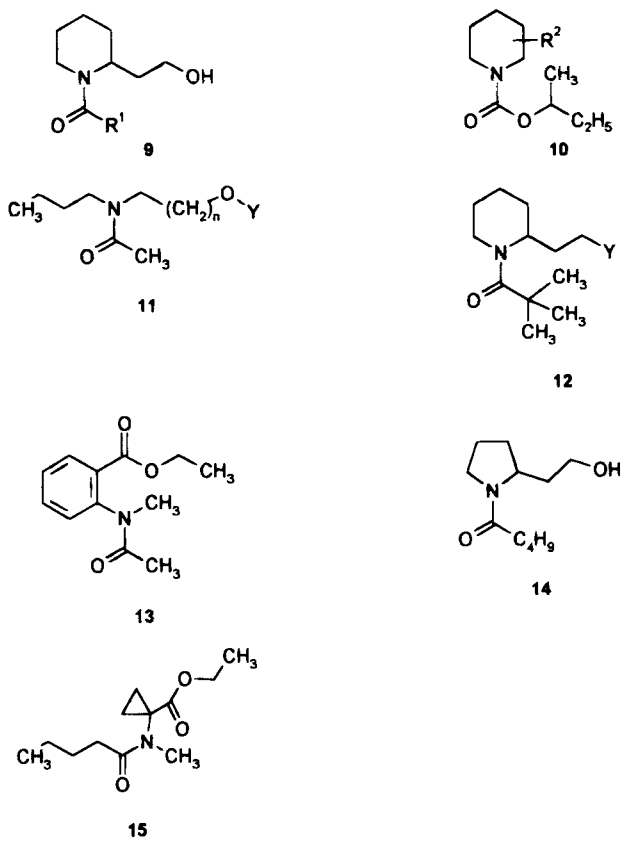

Fig. 2. Structures of the synthesised repellent compounds.

The reaction mixture was stirred for 2 h at 20°C, then water (50 ml) and toluene (100 ml) were added and the organic layer separated and dried over magnesium sulfate. The inorganic material was filtered off, the volatile components removed from the filtrate under reduced pressure and the residue purified by chromatography on silica gel using hexane + acetone (7 + 3 by volume) as eluent.

*2.1.2 1-(Butylaminocarbonyl)-2-(2-hydroxyethyl)piperidine (**9c**)*

n-Butylisocyanate (10 g; 0·1 mole) was added dropwise to a solution of 2-(2-hydroxyethyl)piperidine (13 g; 0·1 mole) in toluene (50 ml) at 20°C. The reaction was strongly exothermic. The reaction mixture was stirred for 1 h at 20°C; the volatile components were then removed and the residue purified by column chromatography on silica gel using methylene chloride + isopropanol (1 + 1 by volume) as eluent. Yield: 20·7 g (91%).

*2.1.3 2-(2-Aminoethyl)-1-(2,2-dimethylpropanoyl)piperidine (**12b**)*

2-(2-Hydroxyethyl)-1-(2,2-dimethyl-propanoyl)piperidine (11 g; 0·05 mole) was heated for 4 h at 50°C with *N*,*N*-dimethylformamide (DMF; 1 ml) and thionyl chloride (7·2 g; 0·06 mole) in chloroform (200 ml).

TABLE 1
Data for Test Compounds of Types **9–12**[a]

Compound	R^1	R^2	*n*	*Y*	*Method*	*Yield* (%)	[^1H] *NMR* (*number of protons*), δ *ppm*
2-(2-Hydroxyethyl)piperidine amides, carbamates and ureas **9**							
9a	C_4H_9-n				a	93	0·93 (3), 1·28–2·0 (13), 2·3–2·4 (2), 2·93 (1), 3·25 (1), 3·5–3·78 (2), 4·0 (1), 4·85 (1)
9b	OC_4H_9-n				a	85	0·93 (3), 1·3–2·05 (12), 2·76 (1), 3·3–3·7 (3), 3·9–4·2 (3), 4·45 (1)
9c	NHC_4H_9-n						0·92 (3), 1·3–1·95 (12), 2·81 (1), 3·21 (2), 3·4 (1), 3·6 (2), 4·05 (1), 4·52 (1), 4·88 (1)
Bis-acylated alkylaminoalkanols **11**							
11a			1	$COCH_3$	a	92	0·96 (3), 1·25–1·40 (2), 1·42–1·61 (2), 2·06–2·13 (6) 3·26–3·37 (2), 3·51–3·59 (2), 4·16–4·23 (2)
11b			2	$COCH_3$	a	82	0·96 (3), 1·22–1·93 (6), 2·06–2·14 (6), 3·18–3·41 (4), 4·06–4·13 (2)
11c			3	$COCH_3$	a	83	0·96 (3), 1·22–1·67 (8), 2·05–2·09 (6), 3·19–3·38 (4), 3·67–4·12 (2)
1-(2-Methyl-propoxycarbonyl)piperidine-amines **10**							
10a[b]		2-(CH_2CH_2OH)			b	84	0·9 (3), 1·2 (3), 1·35–1·8 (9), 1·95 (1), 2·7 (1), 3·4 (1), 3·6 (1), 4·05 (1), 4·8 (1)
10b		3-(CH_2CH_2OH)			b	76	0·9 (3), 1·1–1·2 (4), 1·4–1·7 (8), 1·85 (1), 2·6 (1), 2·9 (1), 3·7 (2), 3·9 (2), 4·73 (1)
10c		4-(CH_2CH_2OH)			b	98	0·95 (3), 1·05 (2), 1·1 (3), 1·45–1·7 (7), 1·85 (1), 2·75 (2), 3·7 (2), 4·15 (2), 4·75 (1)
2-substituted 1,2,2-dimethyl-propanoyl-ethyl-piperidines **12**							
12a				OH	a	87	1·3 (9), 1·5–1·8 (7), 1·98 (1), 2·98, 2·92 (1), 3·23 (1), 3·6 (1), 4·05 (2), 4·85 (1)
12b				NH_2			1·15 (9), 1·25–1·85 (10), 2·6 (2), 3·05 (1), 3·1 (1), 3·25 (1)

[a] For structural formulae see Fig. 2.

[b] Compound **10a** is KBR 3023: m.p. −31°C, b.p. 280°C/1013 hPa, density 1·040 g cm^{-3} at 20°C. Vapour pressure; 3 hPa at 20°C, 17 hPa at 50°C, 23 hPa at 55°C, Viscosity: 135·5 mPa s at 20°C. Rat Toxicity: $LD_{50\,oral}$ >2000 mg kg^{-1}, $LD_{50\,dermal}$ >2000 mg kg^{-1} *Rattus norvegicus* (Berkenhout). Fish Toxicity: LC_{50} >100 mg $litre^{-1}$ (96 h) *Leucisus idus* (L.). Daphnia Toxicity: LC_{50} >100 mg $litre^{-1}$ (48 h) *Daphnia magna* (Straus).

Volatile components were removed under vacuum and the residue purified by chromatography (silica gel; hexane + acetone, 7 + 3 by volume). Yield 11 g (95%). The resulting 2-(2-chloroethyl)-1-(2,2-dimethylpropanoyl)piperidine (6·8 g; 0·029 mole) and potassium phthalimide (5·4 g; 0·029 mole) in ethanol (100 ml) were then heated together for one day under reflux. The mixture was poured into ice-water and processed using standard methods to give 2-(2-phthalimidoethyl)-1-(2,2-dimethylpropanoyl)piperidine in 76% yield. This compound (7·6 g; 0·22 mole) was then heated under reflux for one day together with hydrazine hydrate (1·1 g; 0·22 mole) in ethanol (100 ml). After filtration and distillation (Kugelrohr; b.p. 180°C at 0·1 mbar), compound **12b** was obtained (2·9 g; 43%).

2.1.4 Ethyl N-*acetyl*-N-*methylanthranilate* (***13***)
Ethyl anthranilate (1 mole) was acylated with acetyl chloride (1·1 mole), as in method a. After processing and crystallisation from hexane + methylene chloride, 159·5 g crude ethyl *N*-acetylanthranilate were obtained.

Methyl iodide (15·6 g; 0·11 mole) was added dropwise at 20°C to a suspension of crude *N*-acetylanthranilate (21 g; 0·1 mole) and potassium *tert* butoxide (13·5 g; 0·16 mole) in tetrahydrofuran (100 ml). The reaction mixture was kept for one day at 60°C, then cooled and poured onto ice (800 g). The mixture was extracted with methylene chloride and the organic layer was dried over magnesium sulfate; the methylene chloride was removed by distillation under vacuum and the residue purified by column chromatography (silica gel; cyclohexane + acetone, 8 + 2, by volume). Yield: 9·3 g (42%). [^{1}H]NMR (number of protons) δ ppm: 1·37(3), 1·77(3), 3·20(2), 4·36(2), 7·25–8·02(4).

1-(*N*-Pentanoyl-*N*-methyl)amino-1-ethoxycarbonylcyclopropane,[26] **15**, was prepared as described above except that sodium hydride was used as base (Yield: 60%; b.p. 90–93°C/0·15 mbar). [^{1}H]NMR (number of protons): δ ppm 0·91(3), 1·2–1·9(11), 2·28–2·74(2), 2·95–3·04(3), 4·11–4·22(2).

2.2 Molecular modelling

Conventional modelling techniques were used, involving the program SYBYL.[27] Functional groups were regarded as rigid; the geometries were taken from the Cambridge Structural Database.[28]

2.3 Insects and rearing procedure

2.3.1 Aedes aegypti
The adults were kept in cages (60 × 60 × 55 cm) with netting on the sides and upper part under 12 : 12 h light : dark regime at 27(±1)°C and 70(±10)% relative humidity. A sponge saturated with sucrose solution (100 g litre^{-1}; 'Dextropur'®) was placed on the upper netting as a drinking source. The oviposition stations consisted of beakers (250 ml), half-filled with distilled water and containing rolled filter papers (10 cm long). Guinea pigs were used as host once a week. After oviposition, the filter papers were kept in a bucket (10 litres), the floor of which was covered with a 3-cm layer of humidified cellulose. A half filter paper (from a batch three days up to 21 days old) was placed in a plastic container (40 × 50 cm) with two litres of distilled water and kept in an incubator (29–31°C/60–80% RH) under 12 : 12 h light : dark regime. Food for tropical fish ('Tetramin'®) was added twice daily. After five to six days the population was transferred into plastic dishes (300 ml) containing distilled water to which three plankton tablets ('Plankton'®) were added.

2.3.2 Culex quinquefasciatus, Anopheles stephensi
The breeding was similar to that of *Ae. aegypti*, but the day/night rhythm was changed to 12 : 12 h dark : light. A plastic dish (17 × 20 × 4 cm) was used as an oviposition station, into which a filter paper was placed and the dish then filled to a depth of 2 cm with distilled water. For *An. stephensi*, two twigs of the water plant *Cabomba* sp. were added. One day after oviposition the whole contents were transferred into the plastic containers. *An. stephensi* received twigs of *Cabomba sp.* again. The larvae of both species were fed on plankton food ('Liquizell')® for one day before feeding with 'Tetramin'® was started.

2.3.3 Stomoxys calcitrans
The breeding conditions of the adults were similar to those of the mosquitoes. Beside a drinking station (sponge with 'Dextopur'® in plastic cup) the required volume of blood in the form of a mixture of sodium citrate solution (30 g litre^{-1}; 200 ml) with cattle blood (800 ml) was pipetted into plastic vials (1 cm diam., 10 cm long) which were closed on one side by a rubber stopper. The vials were fixed, upside down, into holes in the ceiling of the cage and were replaced every day. The four- to five-day-old females began egg laying on a humidified substrate of powdered grass and manure (1 + 1) in a plastic dish. Three days later this was covered by feeding material (1 cm high) which consisted of powdered grass (225 g), wheat bran (450 g), yeast (15 g) and malt (40 g), mixed with water to a certain degree of wetness that was maintained during the larval development. After 12 to 16 days the larvae pupated at the upper edge of the dish. The fresh puparia were gathered, dried under room conditions for one day and stored in a refrigerator (for up two weeks).

2.3.4 Periplaneta americana
The insects were kept in plastic containers (80 × 60 × 50 cm) at 27(±2)°C under a 12 : 12 h light : dark regime, fed with commercial dry dog food

and supplied with water *ad libitum*. Pieces of cardboard served as hiding places. The specimens lived in colonies comprising both sexes and all nymphal stages. Before experimentation, adult males were kept separately in order to prevent adaptation to pheromones.

2.4 Biological test methods

2.4.1 *Efficacy on guinea pigs*

The insects were kept as active biting populations in meshed test cages (60 × 60 × 55 cm) under the conditions described in Section 2.3.1. New adult mosquitoes were added once a week, to maintain constant population size and biting activity. All insects were fed at drinking stations as described above. In the case of *St. calcitrans*, the insects were completely replaced after two days because they began to die as a consequence of the absence of a blood meal. An area of 50 cm^2 of a guinea pig's back was shaved and then treated with depilatory cream, the cream then being removed with water. One day later the animal was fixed in a narrow cage (box) so that only the shaved area was accessible to the insects. The compounds to be tested were dissolved in ethanol to give the desired concentration. The shaved area was treated with this solution (0·4 ml), using a pipette, and the solvent allowed to evaporate, after which the guinea pig was exposed to the insects. The number of bites occurring over five minutes was recorded and the animal was removed from the cage. The test was repeated one hour later. This was continued until the number of bites per five-minute period declined to five. After the trial the guinea pig was cleaned with a cloth containing acetone and used again three weeks later. Only one guinea pig was used in initial tests but the final tests involved 12 guinea pigs from which the mean protection time (h) and standard deviation were calculated. For statistical analysis, Student's *t*-test[29] was used.

2.4.2 *Behavioural tests*

2.4.2.1 Mosquitoes. Mature female *Ae. aegypti* from a colony, maintained at Bayer AG, Monheim, were tested in a Y-shaped wind tunnel, illustrated in Fig. 3. Each end of the Perspex Y-tube had a removable chamber. The start chamber with 20 mosquitoes was attached to the stem of the Y-tube. Both chambers on the branches of the Y-tube fitted into the tubes where the odour stimuli were presented. The synthetic odours were produced by blowing purified air through a glass cartridge containing the odour solution on its inner surface. Behind the glass cartridge outlet, a human hand could be held into the air stream of the Y-tube *via* a lateral opening. A permanent air stream (cleaned by a charcoal filter, adjusted to 70%(±5)% RH and heated to 28°C(±1)°C) flowed through the whole system. The wind speed in the tunnel was 0·2–0·3 m s^{-1} in the branches and 0·4–0·6 m s^{-1} in the stem of the Y-tube.

Groups of 20 females were enclosed in the start chamber. The hand (as a standard for host body odours) and cartridges with odour-producing compounds were brought into the air current in the test arm of the tunnel. Purified air, or glass cartridges containing only solvent, in the other arm of the tunnel served as control. The number (or percentage) of the mosquitoes

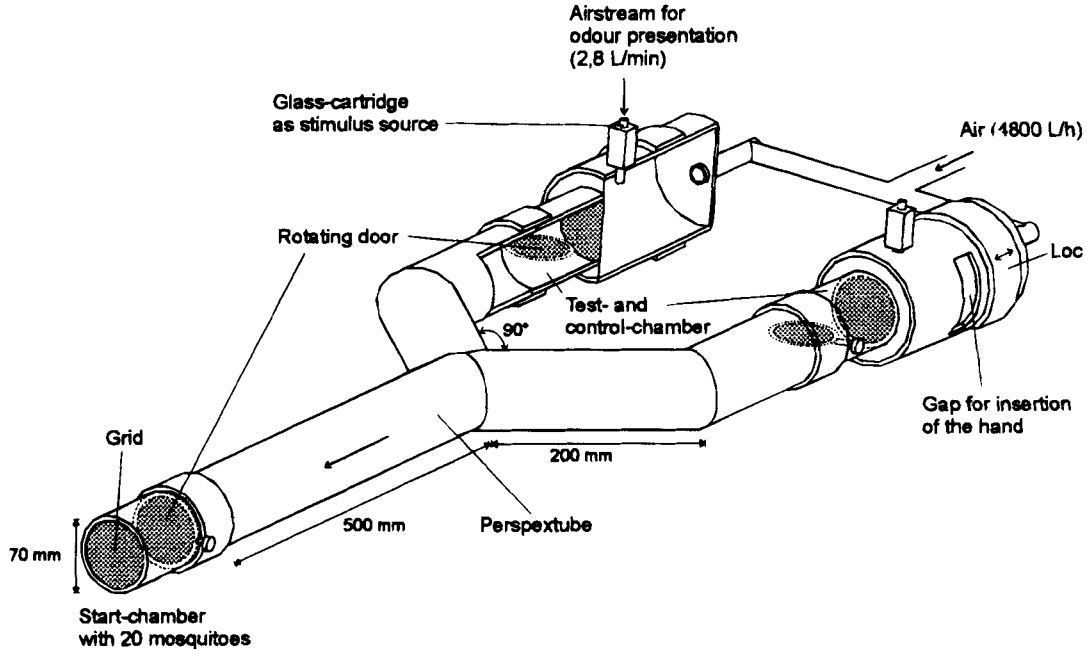

Fig. 3. Y-tube wind tunnel: schematic drawing of the wind tunnel and arrangement for application of the odour stimuli.

leaving the start chamber during stimulation was taken as a measure of 'activation'. The number (percentage) of insects trapped in the test chamber on a grid in front of the stimulus was taken as a measure of 'attraction'. Each trial consisted of 11 replicates.

2.4.2.2 Cockroaches. Adult male *P. americana* were isolated from females for at least two weeks. Experiments were performed with groups of five individuals in a total of ten 15-litre plastic basins, 2–6 h after the start of the daily scotophase. After introduction of a glass tube containing a filter paper ('odour cartridge') soaked with a solution of periplanone-B, a major component of the sex pheromone, and blowing purified air through the cartridge, the males displayed characteristic sexual behaviour, including antennal movements and running activity dependent on the amount of pheromone at the odour source. The frequency of the males' crossing the centre line of the cage during one minute was taken as a measure of male locomotion activity (counts) (Fig. 4).

Male locomotion activity was determined in response to mixtures of periplanone-B and experimental repellent compounds. The periplanone-B charge was kept constant at 0·1 pg, while the repellent amounts were varied. The mean response to periplanone-B stimuli was 100 counts. Count values < 100 indicated an inhibitory effect on male periplanone-B -induced activity.

2.4.3 Electrophysiological tests

The experimental specimens of female *Ae. aegypti* and male *P. americana* were fastened to a holder and the antennae immobilised by tape. Glass or tungsten microelectrodes with tip diameters of about 1 μm were inserted into the sockets of individual olfactory sensory organs ('sensilla') on the antennae. Recorded impulses were fed *via* a high-impedance pre-amplifier into a PC for sampling and impulse discrimination under aspects of cell specificity and dosage–response characteristics. Odour stimuli were administered by blowing purified air through glass tubes (50 mm × 4 mm inner diameter) containing odour-soaked filter paper towards the area of the recorded sensillum. Empty or solvent-filled tubes served as controls.

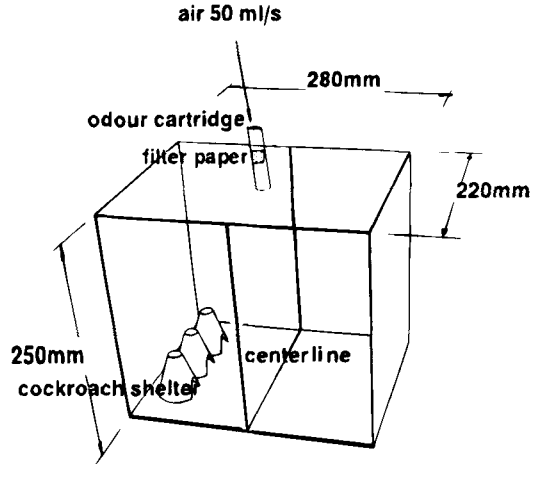

Fig. 4. Apparatus for measuring male cockroach activity.

2.5 Biochemical tests

2.5.1 Materials

Adult male cockroaches (*P. americana*) were obtained from local suppliers. Radioligand assay kits for cAMP and inositol triphosphate (IP_3) were purchased from Amersham, UK. Periplanone-B was a generous gift from Prof. J. Boeckh, Regensburg, Germany. All other chemicals were of analytical grade and purchased from Sigma. For stimulation experiments with pheromones, stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted with stimulation buffer (assay-buffer supplemented with sodium cholate (0·5 g litre^{-1}), ATP (1 mM), GTP (2 μM) and appropriate calcium chloride to provide a free calcium concentration of 0·012 μM) to obtain the experimental concentrations. The solutions were mixed in an ultrasonic water-bath and used immediately. Control experiments involved application of buffer containing the highest DMSO concentration, which did not exceed 0·5 g litre^{-1}, at which concentration DMSO did not affect the reaction.

2.5.2 Methods

2.5.2.1 Tissue preparation. Tissue was prepared as described previously.[30] Briefly, living insects were cold-adapted (4°C) for at least 3 h. Antennae and muscle were dissected and homogenised in assay buffer (MOPS 50, sodium chloride 200, EGTA 10, magnesium chloride 2·5, DTT 1 mM; pH 6·9). The homogenate was centrifuged at 800*g* for 15 min and the supernatant used immediately.

2.5.2.2 Measurements of rapid changes in second-messenger concentrations. A rapid quench device with three syringes was used to measure stimulus-induced changes in concentrations of second messengers as described previously.[30] Briefly, syringe I contained the antennal fraction in assay buffer and syringe II was filled with stimulation buffer containing the pheromone sample, which was dissolved as described in Section 2.5.1. All solutions were maintained at 30°C. Syringe III contained perchloric acid (70 g litre^{-1}) cooled to 0°C. The reaction was started by mixing the contents of syringes I and II and quenched with perchloric acid from syringe III. Activation of the three syringes was controlled by an IBM AT computer. The quenched samples were collected and stored on ice before determining the second-messenger concentrations as described previously.[31]

3 RESULTS AND DISCUSSION

3.1 Molecular modelling

The detailed biochemical steps leading to the desired repellent effect, especially the 3D-structure of the molecular receptor(s) and the local reaction with the repellent are not known. This is not the optimal basis for molecular modelling studies. Nevertheless, we supported the chemical efforts by developing a general structural framework with high probability for repellent activity. This model does not claim to explain the underlying biochemistry, but is a purely operational model to give some guidance for the synthesis work.

We started from the defined set of repellents, i.e. **5**, **11a**, **12a**, **13** and **14**. These structures have a pattern consisting of an amide group, an sp^3 hybridised oxygen atom within a hydroxyl or ester group and a lipophilic core linking the two functional groups. Comparison with **2**, a compound with an ester instead of amide group, suggested that the essential feature in the amide group was the carbonyl oxygen atom. Figure 5 shows these moieties for **12a** schematically:

All compounds in the set have a more-or-less flexible linkage between the two functional groups. For each molecule this flexibility may lead to a large set of energetically possible relative arrangements of the carbonyl oxygen atom (mostly in the amide group) and the sp^3 hybridised oxygen atom. The superimposition of the oxygen atoms themselves is not a necessary condition for a common interaction with a receptor. Therefore we allocated lone pairs to the oxygen atoms and extended the distance between oxygen atom and lone pair to 2 Å, the typical length in the range between a hydrogen bridge and a bond in an organometallic complex. The lone pairs indicate the directionality of binding to other polar groups. To further simplify the approach, we replaced the lone pairs at each oxygen atom with their centroid, using these centroids as the relevant attachment points. The idea was to find a common arrangement of the two attachment points, including a similar directionality of the binding, over the complete set of structures, to postulate this structural motif as relevant for the repellent action and to verify the operational model by mapping new active, as well as inactive, compounds onto it.

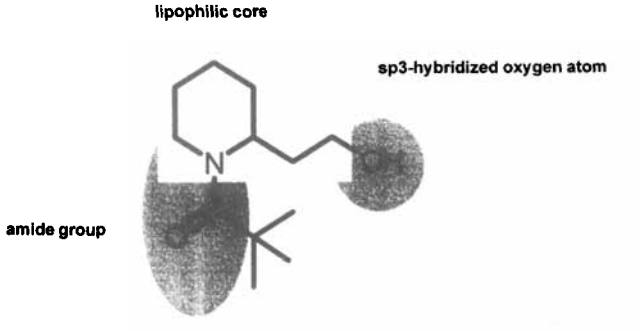

Fig. 5. Structural pattern leading to repellent activity.

In fact, it turned out that there was just one family of molecular geometries that fulfilled all the requirements. At the same time, the model suggested that certain compounds should demonstrate no repellent effect, although chemically they differed only little from active compounds.

The type of conformation identified was refined stepwise by including other compounds, e.g. **15**, leading to the model in Plate 1. The model shows some characteristic features:

- the essential binding points, i.e. the two oxygen atoms represented by their lone pair centroids (colour codes in yellow) in a well-defined distance and orientation (the equivalent functional groups are colour-coded in green and red, respectively);
- a lipophilic moiety linking the two attachment points which may be of considerable volume, although we identified some forbidden spatial regions;
- a lipophilic moiety beyond the carbonyl group, which turned out to be of high relevance for tuning the size of the repellent effect.

The lipophilic moiety beyond the carbonyl group could be explored by introducing hetero atoms to stabilise specific conformations and different lipophilic substituents to vary the volume. The result is shown in Plate 2, where **10a** (KBR 3023) is shown in conjunction with the surface of the accessible volume.

This model, together with other boundary conditions, e.g. with respect to volatility, was used to design possible candidate repellent structures.

3.2 Biological tests

3.2.1 Efficacy on guinea pigs

Some structure–activity relationships are shown in Table 2, where the protection time (in hours) as a measure for repellent activity for different acylated α,ω-aminoalcohols is compared to the activity of the standard, deet (**1**).

2-(2-Hydroxyethyl)piperidines which have *N*-alkoxycarbonyl or *N*-alkanoyl groups possess high activity against bloodsucking insects. From unpublished experiments we know that alkoxyalkyl groups in the acyl chain would have led to loss of biological activity. Among piperidines with heteroatoms in the side chain, compounds with oxygen in position 2 have high activity, whereas compounds with nitrogen are inactive. With the open-chain aminoalcohols **11**, there was a maximum activity with three carbon atoms between nitrogen and oxygen, which was in good agreement with our modelling studies.

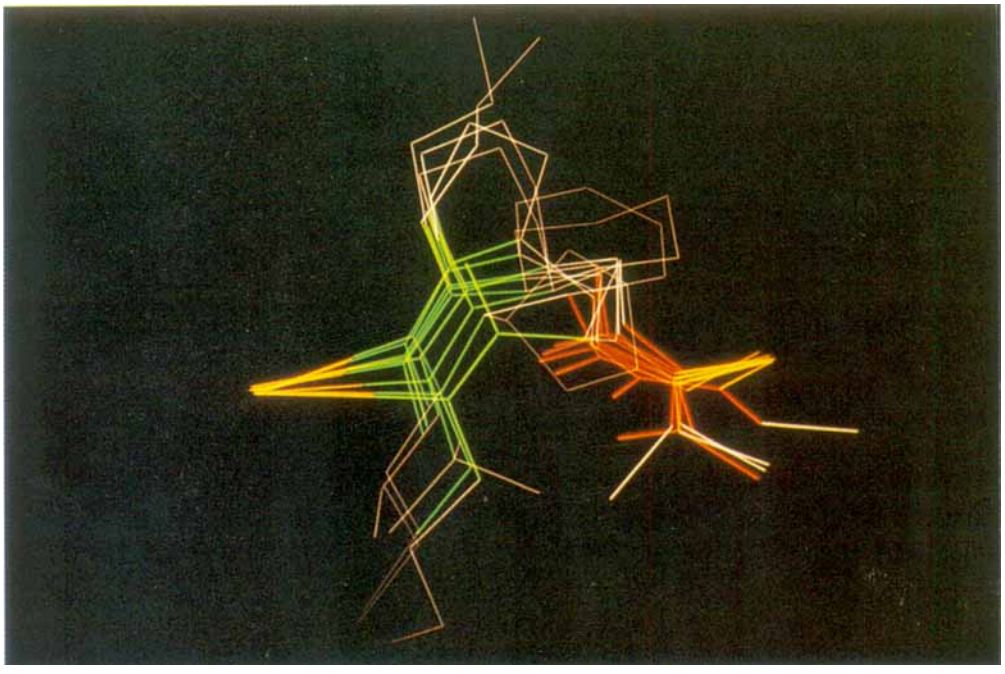

Plate 1. Operational 3D-model for the design of repellents.

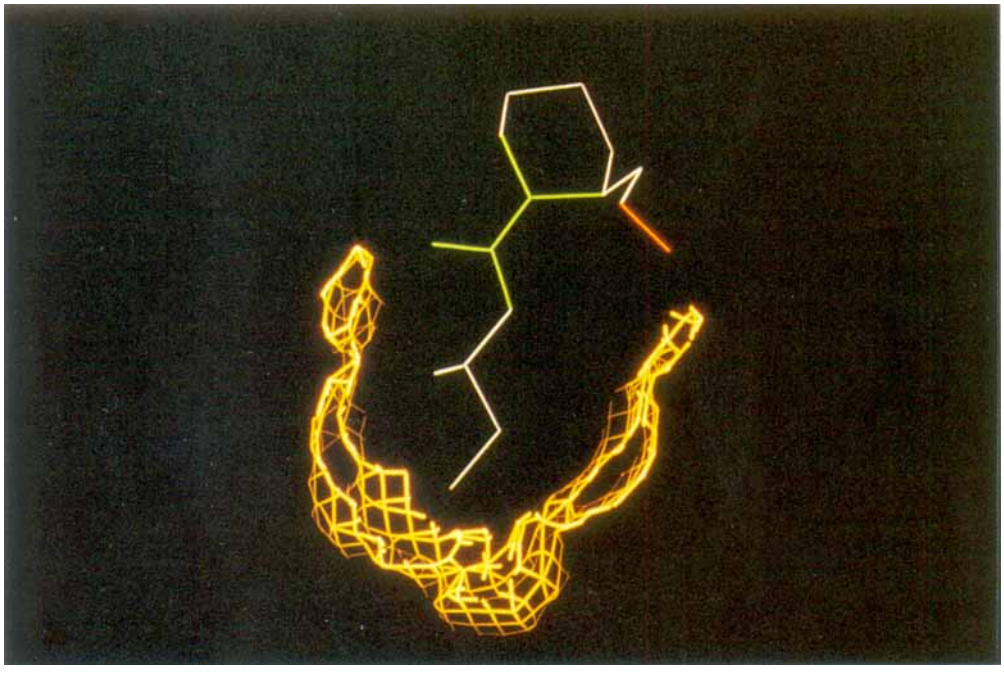

Plate 2. Shape of lipophilic pocket as derived from comparison of active with inactive structures.

TABLE 2
Repellent Activities of Test Compounds of Types **9–12**[a,b]

Compound	R^1	R^2	*n*	*Y*	*Repellent activity duration (h)*
2-(2-Hydroxyethyl)piperidine amides, carbamates and ureas **9**					
9a	C_4H_9-n				10 (6)
9b	OC_4H_9-n				>12 (6)
9c	NHC_4H_9-n				No activity
Bis-acylated alkylaminoalkanols **11**					
11a			1	$COCH_3$	4[c] (6)
11b			2	$COCH_3$	7 (3)
11c			3	$COCH_3$	10[c] (6)
1-(2-Methyl-propoxycarbonyl)piperidine-amines **10**					
10a		2-(CH_2CH_2OH)			>9 (5)
10b		3-(CH_2CH_2OH)			No activity
10c		4-(CH_2CH_2OH)			No activity
2-substituted 1,2,2-dimethyl-propanoyl-ethyl-piperidines **12**					
12a				OH	>11 (5)
12b				NH_2	No activity

[a] For structural formulae see Fig. 2.
[b] Corresponding figures for deet (**1**) are in parentheses.
[c] Sometimes not 100% protection.

The most promising compound **10a** (KBR 3023) was tested for broad-spectrum efficacy (Table 3).

Against all insect species tested, **10a** gave statistically significantly longer protection than **1**. *C. quinquefasciatus* was more susceptible to both compounds than *Ae. aegypti*, which itself was more easily repelled than *An. stephensi*.

This differential sensitivity of different mosquito species to **1** agrees with the results of several investigators. Curtis *et al.*[32] showed that, in the field, *C. quinquefasciatus* is more susceptible than most anopheline species, to deet-impregnated anklets. Frances *et al.*[33] reported that, in laboratory tests on human forearms, *An. dirus* (Peyton & Harrison) is much more tolerant to **1** (and to dimethyl phthalate) than *Ae. albopictus* (Skuse). Rutledge *et al.*[34] showed that, on membranes treated with **1**, the ED_{50} values for *An. albimanus* and *An. stephensi* were higher than for *Ae. aegypti*, which was again more tolerant than *Culex pipiens* (L.). According to Sikder *et al.*,[35] *C. quinquefasciatus* also reacts more sensitively to other repellents (*N,N*-diethyl-*p*-tolylacetamide, *N,N*-diethyl-*m*-tolylacetamide) than *Ae. aegypti*.

The efficacy of **10a** was confirmed in our field tests against *Ae. aegypti*, *Ae. Albopictus*, *C. quinquefasciatus*, *St. calcitrans* and the tick *Ixodes ricinus* (L.) (unpublished results).

TABLE 3
Protective efficacy of Compound **10a** (KBR 3023) in Comparison to **1** (deet) against Different Bloodsucking Diptera on Guinea Pigs[a]

Species	*Compound*[b]	*Protection time (h)* (±*SD*)	*P*
Ae. aegypti	**1** (30)	3·6 (±1·0)	
	10a (30)	7·2 (±1·0)	<0·01
C. quinquefasciatus	**1** (30)	6·8 (±1·8)	
	10a (30)	12·2 (±1·8)	<0·01
An. stephensi	**1** (30)	2·7 (±1·2)	
	10a (30)	6·4 (±1·1)	<0·01
St. calcitrans	**1** (50)	2·4 (±1·6)	
	10a (50)	9·3 (±2·5)	<0·01

[a] Mean of 12 replicate experiments.
[b] Compound applied as an ethanolic solution; concentration (g litre^{-1}) in parentheses.

3.2.2 Behavioural tests with Aedes aegypti: *Interference of compounds 1 (deet) and 10a (KBR 3023) with attractants*

Clear and dosage-dependent effects of **1** and **10a** become apparent as soon as behaviourally active odorants and the corresponding responses are studied. Then a reduction of 'activity' and 'attraction' is observed in the presence of **1** or **10a** (Fig. 6, Tables 4, 5). In Fig. 6, 85% of the animals leave the start chamber (= 'activity') when a human hand is exposed into the airstream of the test arm; 78% arrive at a grid in front of the hand (= 'attraction') and only 8% arrive in the control arm. When **1** is added the activity, as well as the attraction, is reduced with increasing amounts of **1** (cf. abscissa). At high doses of **1** the number of animals

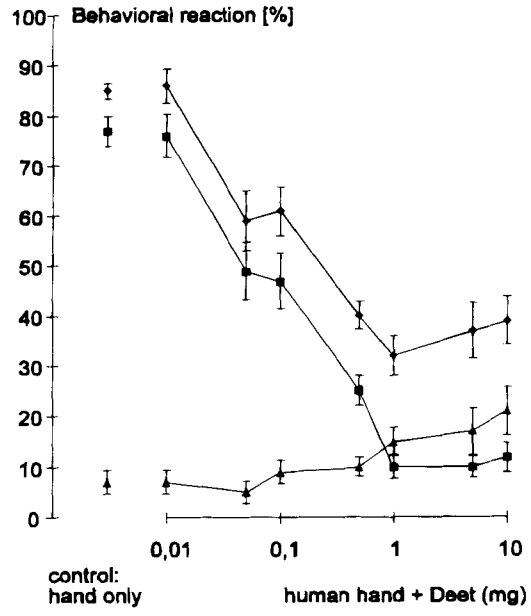

Fig. 6. Reducing effect of compound **1** (deet) with attractive odours emanating from a human hand in the Y-tube wind tunnel on female *Aedes aegypti.* (—◆—) Activity; (—■—) Attraction test chamber; (—▲—) Attraction control chamber

arriving in the control arm is slightly increased. Table 4 shows such effects side by side for **1** and **10a**.

Interestingly, the effects of **1** and **10a** are qualitatively similar in reducing the effects of such different attractants as carbon dioxide and lactic acid for *Ae. aegypti.* Both compounds are components of the body emanations of hosts (Table 5).

3.2.3 Behavioural tests with Periplaneta americana

The running activity of male cockroaches elicited by female pheromone is even increased slightly by addition of small amounts of **1** (deet) and **10a** (KBR 3023) before it is decreased at higher doses of the compound (Fig. 7). A charge of 0·1 pg periplanone-B in the source raised the locomotion activity of male *P. americana* to about 100 activity counts. This response was strongly reduced if higher doses of **1** and **10a** were mixed with the pheromone.

3.2.4 Possible mechanisms of the actions of compounds 1 (deet) and 10a (KBR 3023)

A reduction of the activating or attracting effect of an odour source by **1** or **10a** could be based upon one or more of the following mechanisms:

(a) a general depression or impairment of the locomotory behaviour of the insect through a general, possibly pharmacological, effect upon the nervous system.[36] However, there is no indication as yet for such effects.

(b) an antagonistic and depressing effect upon the insect's receptors for the host odours or the pheromone respectively. There is evidence from electro-

TABLE 4

Effects of Compounds **1** (deet) and **10a** (KBR 3023) on Female *Aedes aegypti:* Reduction of Activity and Attraction of the Odours of a Human Hand[a]

Stimulus in test arm	*Leaving star chamber* (%) (±*SD*)	*Arriving in test chamber* (%) (±*SD*)	*Arriving in control chamber* (%) (±*SD*)
Hand	97 (±4)	93 (±6)	4 (±5)
Hand + 0·1 mg **10a**	76 (±13)	49 (±11)	15 (±10)
Hand + 0·1 mg **1**	59 (±17)	36 (±18)	12 (±10)
Hand + 1 mg **10a**	72 (±16)	42 (±18)	18 (±14)
Hand + 1 mg **1**	50 (±18)	26 (±13)	16 (±9)

[a] Mean of 11 replicate experiments.

TABLE 5

Effects of Compound **1** (deet) on Female *Aedes aegypti:* Reduction of Activity and Attraction Elicited by the Odours of Lactic Acid and Carbon Dioxide[a]

Stimulus in test arm	*Leaving start chamber* (%) (±*SD*)	*Arriving in test chamber* (%) (±*SD*)	*Arriving in control chamber* (%) (±*SD*)
Lactic acid (4 μg)	58 (±10)	32 (±7)	3 (±4)
Lactic acid + 1 mg **1**	21 (±12)	1 (±2)	4 (±5)
CO_2 (6% by volume)	80 (±17)	51 (±15)	3 (±3)
CO_2 + 1 mg **1**	22 (±8)	1 (±2)	4 (±6)
Hand	97 (±5)	94 (±7)	3 (±5)
Hand + 1 mg **1**	40 (±8)	20 (±6)	6 (±5)

[a] Mean of 11 replicate experiments.

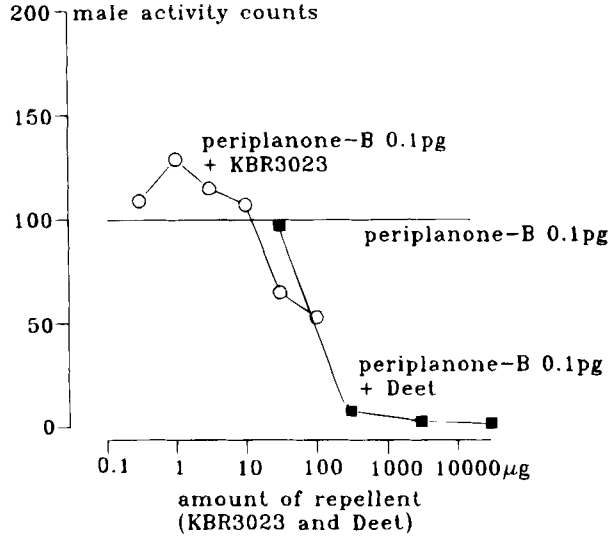

Fig. 7. Reducing effect of compounds **1** (deet) and **10a** (KBR 3023) on the pheromone-induced locomotion activity of male *Periplaneta americana.*

physiological experiments on host odour receptors of *Ae. aegypti* that addition of deet to lactic acid, a stimulating component of the host odour, reduces or inhibits the responses of the receptor cells involved.[37] This kind of effect has not been investigated for other attractants such as carbon dioxide.

(c) an alteration of the olfactory input, in the sense that the host-characteristic response pattern of the corresponding receptor cell population is changed into a different pattern which 'masks' the original pattern. Evidence for this alternative has been demonstrated in *Ae. aegypti* as well as in *P. americana* in the experiments discussed in Section 3.3.

3.3 Electrophysiological observations

3.3.1 Aedes aegypti

The receptor cells for host emanations are found in a certain type of sensory hair, the so-called A-3 type sensilla on the antennae as shown in the SEM prints in Fig. 8. There was no response of such cells to **1** or **10a**. However, cells contained in sensilla of the type A-2 were strongly and specifically excited by the two substances, each of which was effective in a different way (Fig. 9). Two cells were discriminated according to different impulse amplitudes. Only the cell with the smaller amplitude is excited by **10a**, whereas both cells respond to **1**.

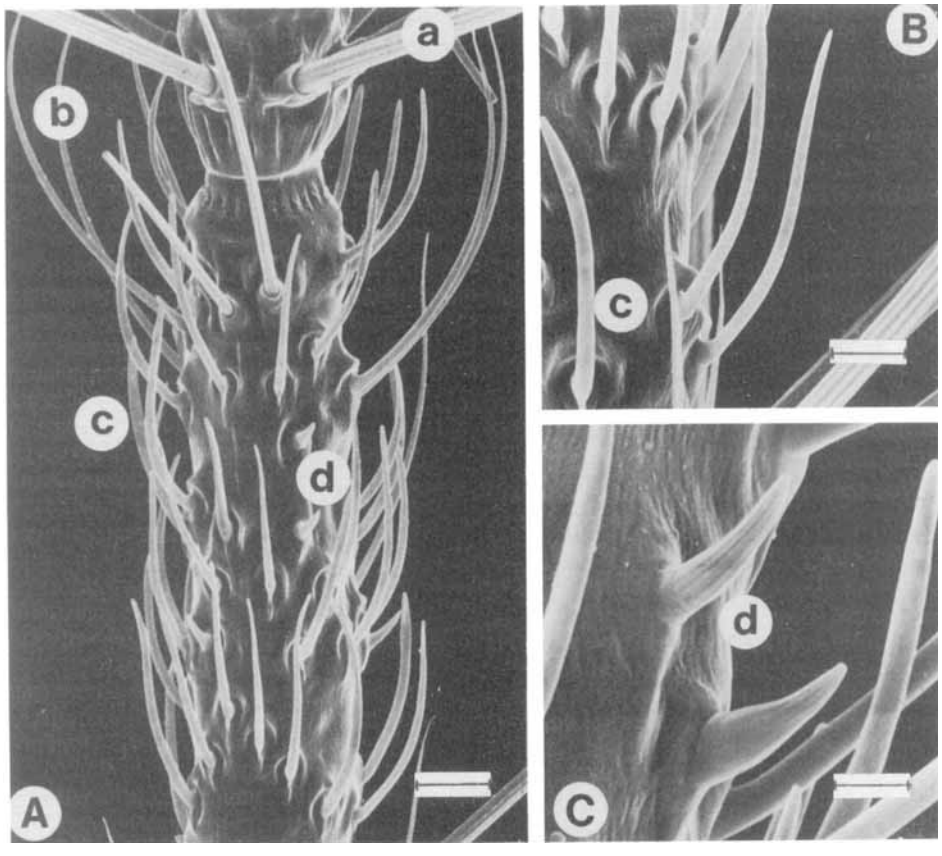

Fig. 8. SEM electron micrograph of a female *Aedes aegypti* antenna. A: one of the total of 13 segments of the antenna with different morphological types of sensory hairs (sensilla) a, b, c, d, B and C: details. (Scale bar Fig. 8A: 20 µm, Fig. 8B: 10 µm, Fig. 8C: 5 µm). Type A-2 sensilla (c) contain receptor cells which are sensitive to **1** (deet) and/or **10a** (KBR 3023) but not to host odours. Type A-3 sensilla (d) contain cells that respond to human body odours and several of its components including e.g. lactic acid.

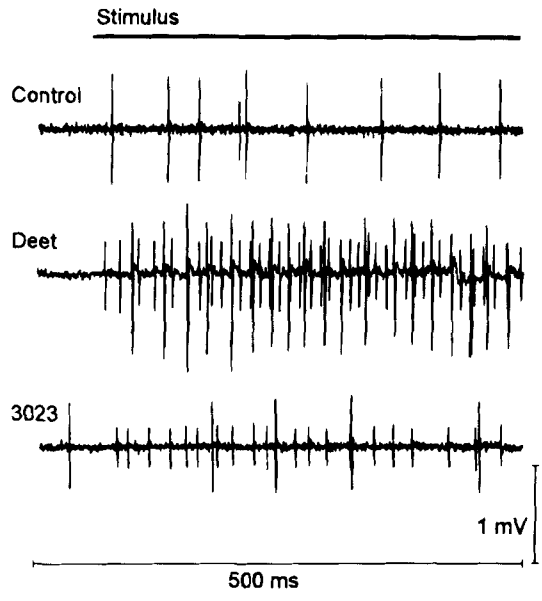

Fig. 9. Microelectrode recordings from an A-2 type sensillum of female *Aedes aegypti* (cf. Fig. 8A, B, C) exposed to compounds **1** (deet) (50 mg) and **10a** (KBR 3023) (50 mg).

3.3.2 Periplaneta americana

The receptor cells are found in a certain type of sensory hair, the so-called sw-B sensilla on the antennae. Figure 10 is an electron micrograph of a male *P. americana* antenna, showing the position of the recorded sensillum.

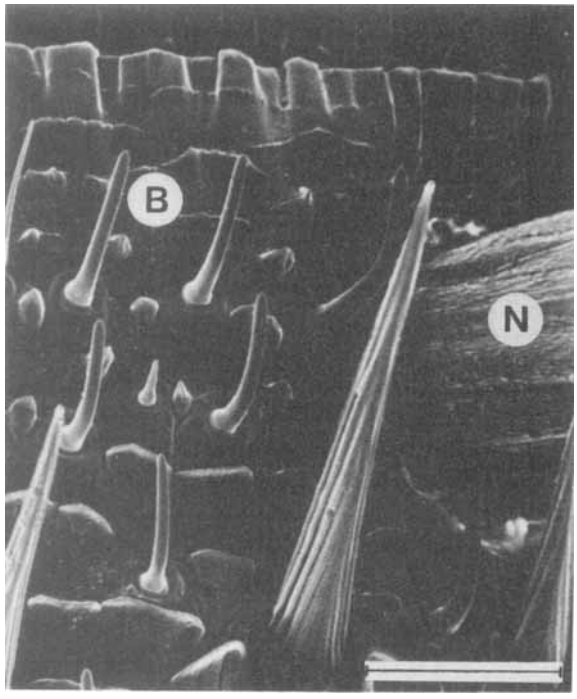

Fig. 10. SEM electron micrograph of a male *Periplaneta americana* antenna (scale bar: 20 μm) Exposure of one of the total of 150 segments. A cactus needle (N) was inserted near the recorded sensillum to indicate its position within the surrounding pattern of sensilla. The sensillum belongs to sw-B-type sensilla (B) and houses (i) a pair of receptor cells sensitive for compounds from the female pheromone and (ii) another pair of receptor cells sensitive to other substances, e.g. terpenoid compounds.

The two 'pheromone cells' never became activated or inhibited by **1** or **10a**. One of the second pair of cells responded readily to **1** and to **10a** (Fig. 11).

3.3.3 Conclusions from electrophysiological tests

These electrophysiological data suggest that both **1** and **10a** act specifically upon certain olfactory receptor cell types that are not involved in the perception of the odours relevant during attraction in the two insects. This is well in accordance with the results of the biochemical tests (Section 3.4.): **10a** triggers an intercellular signal cascade in antennal tissue of cockroaches which is characteristic for specific chemosensory responses rather than for pharmacological or 'general' chemical or suppressive effects. Therefore, their counteraction against the natural attractants is due to perception by other cell types and will take place at the level of the CNS. This could be in addition to a direct inhibitory effect on cells like the lactic acid receptor (Section 3.2.4). The two compounds probably provide multipotent stimuli which override the odour-specificity of all kinds of olfactory receptor cells or overstimulate them. They are not, however, mimics of one certain odorant which plays a given role in a certain biological context in one species. They might just 'fit' into the odour spectra of certain types of olfactory cell (perhaps into their 'side' spectra) and be sufficiently potent to stimulate them in a physiological manner which is not poisoning or narcotising. Such inputs, together with the ones from the 'normally' excited receptors for the host or the female odour, may summate to a new input pattern in the olfactory brain which the latter does not 'understand' in terms of host or female.

3.4 Biochemical tests

To investigate its specificity and mode of action, the potential of **10a** (KBR 3023) to affect second-messenger systems in antennal preparations from male *P. americana* was analysed.

Using a fast kinetic methodology, these preparations were stimulated with the species-specific pheromone periplanone-B or with **10a** for 50 msec. As shown in Fig. 12, a concentration of 600 pM of the pheromone induced a significant increase in the concentration of inositol triphosphate (IP_3) (Fig. 12A), whereas the level of cyclic AMP remained unchanged (Fig. 12B). The compound **10a** also elicited selectively an IP_3 response in antennal preparation from cockroach. Upon application of 100 μM **10a** IP_3 increased from 128(±35) to 310(±71) $pmol^{-1}$ mg protein; there was no change in the concentration of cAMP.

Thus the repellent odorant **10a** activates the same second-messenger pathway in cockroach antennae as the pheromone. However, the **10a** responsive system is much less sensitive; the stimulating active doses of the repellent have to be orders of magnitude higher than the pheromone.

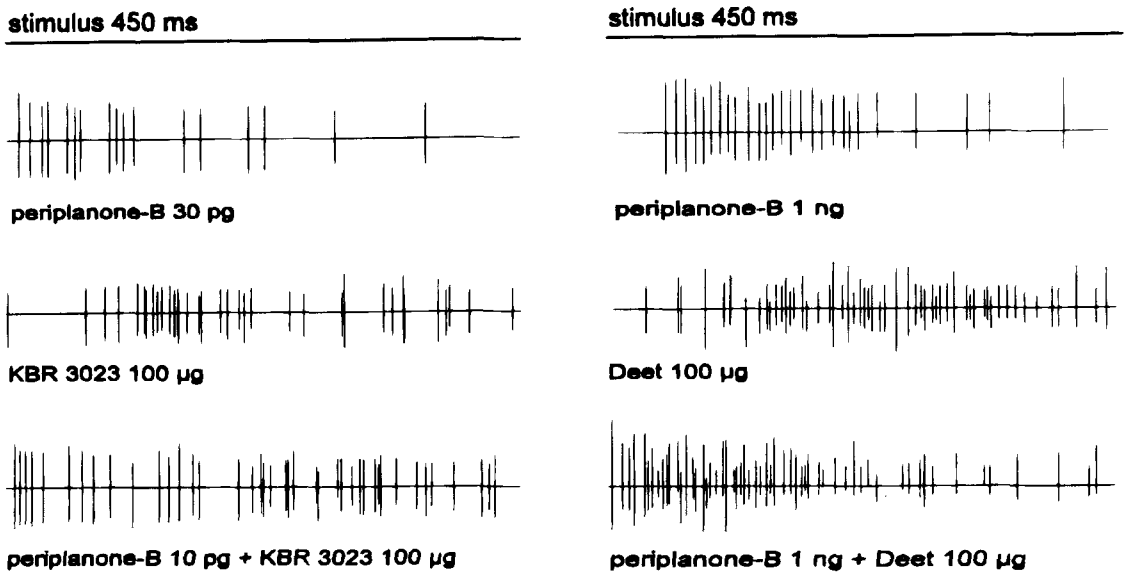

Fig. 11. Microelectrode recordings from two sw-B type sensilla of male *Periplaneta americana* (cf. Fig. 10) exposed to periplanone-B, **1** (deet) or **10a** (KBR 3023) and a mixture of pheromone and repellent.

In order to determine the tissue-specificity of the **10a**-induced second-messenger signal, similar experiments were performed on preparations from muscle tissue. As depicted in Fig. 13, application of different concentrations of **10a** induced a dose-dependent increase in IP_3 concentration in antennal preparations, whereas the IP_3 level in muscle tissue was not affected by the repellent.

The selective activation of the IP_3 level pathway in antennal preparations suggests that KBR 3023 may interact with subsets of G-protein coupled receptors in antennal sensory neurones thereby triggering a transduction cascade and exciting the selfsame.

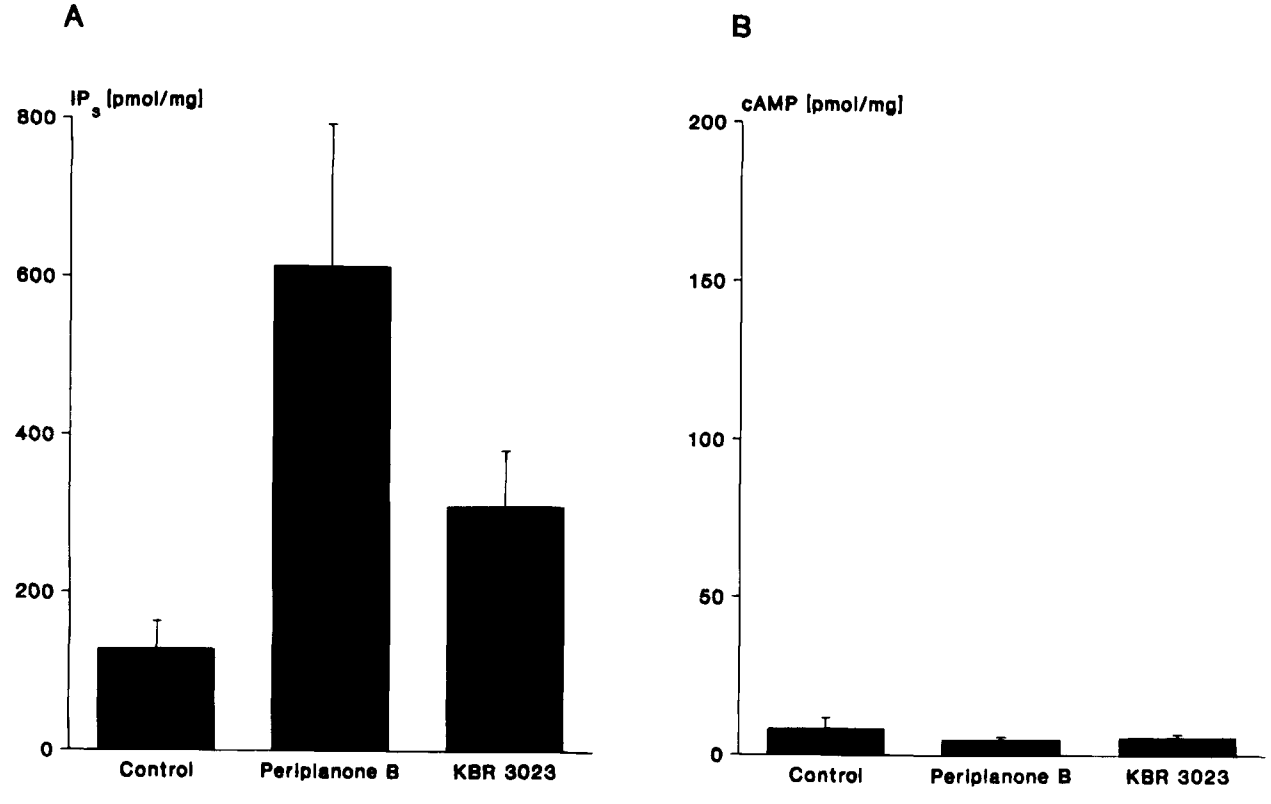

Fig. 12. Comparison of cAMP and inositol triphosphate (IP_3) concentration in antennal preparations from *Periplaneta americana* upon odorant stimulation. Antennal preparations were stimulated with 600 pM periplanone-B or 100 µm **10a** (KBR 3023) for 50 msec; subsequently the concentration of cAMP and IP_3 was determined. Only the level of IP_3 was elevated after odorant stimulation (A); the concentration of cAMP was not affected (B).

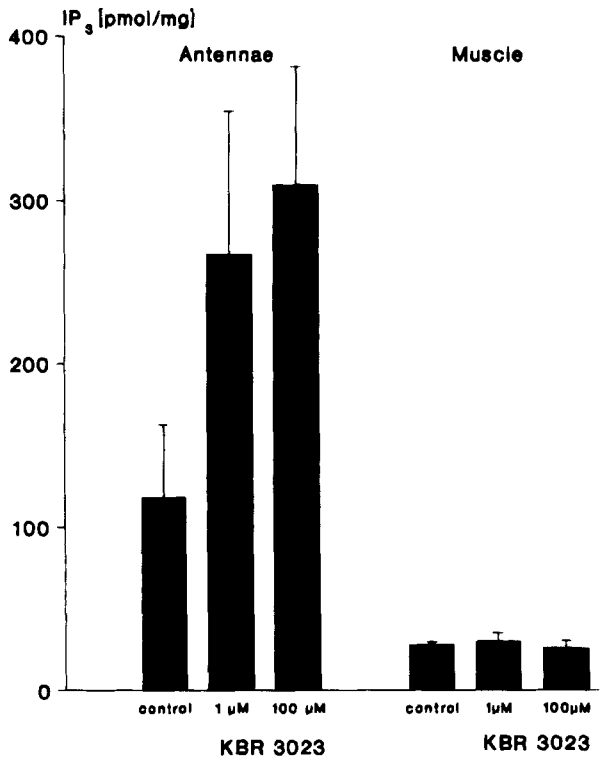

Fig. 13. Tissue-specificity of compound **10a** (KBR 3023)-induced formation of inositol triphosphate. Whereas in antennal preparations application of 1 µM and 100 µM of **10a** resulted in a dose-dependent rise in the concentration of IP_3, stimulating muscle tissue from cockroaches with **10a** did not induce any change in the level of IP_3.

4 CONCLUSIONS

After considering all the relevant properties such as biological activity, cost of raw materials, cosmetic characteristics and the influence on plastic materials we decided to develop the carbamate **10a** (KBR 3023) as a new insect repellent.

All the toxicological studies were carried out according to the requirements of the US EPA, with whom the registration will subsequently be filed.

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